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Stem diameter variations and cold hardiness in walnut trees

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Abstract

The effect of freezing temperatures on stem diameter was measured in the field and in climatic chambers using linear variable differential transformers (LVDT sensors). In acclimated stems, there was reversible stem shrinkage associated with freeze–thaw cycles. The maximum shrinkage correlated with stem diameter (thickness of the bark). The wood was responsible for only 15% of the shrinkage associated with a freeze event, and experiments with isolated bark showed that connection with the wood was not necessary for most of the freeze-induced shrinkage to occur. Considering the amount of stem shrinkage associated with summer drought in walnut, the amount of contraction of the bark with freezing was actually much less than might be predicted by water relations theory. Reversible stem shrinkage occurred in living tissues, but not in autoclaved tissues. For the latter, swelling was observed with freezing and this swelling could be explained by the bark alone. Similar swelling was observed during September and October for non-acclimated plants. Water was lost with each freeze–thaw cycle starting with the first, and freezing injury of the bark, with discoloration of tissues, was also observed in non-acclimated plants. Given that the diameter fluctuation patterns were dramatically different for acclimated versus non-acclimated plants, and for living versus autoclaved tissues, LVDT sensors could represent a novel, non-invasive approach to testing cold hardiness.

Introduction

It is commonly recognized that low temperatures are a major limiting factor that may explain species distribution in cold climates (Parker, 1963; George *et al.*, 1974). For this reason, freezing tolerance is often a selection criterion in breeding programmes (Stushnoff, 1972; Ashworth and Wisniewski, 1991; Palonen and Buszard, 1997) and several research programmes have placed a primary emphasis on elucidating mechanisms of freezing injury and cold acclimation (Ashworth, 1986; Rodrigo, 2000). Following a frost, the fate of the cell depends on where the formation of ice crystals takes place (Mazur, 1969). Thus, ice formation can be either intracellular which is fatal and causes cell death if cooling is rapid, or extracellular which protects the cells themselves at least temporarily (Rodrigo, 2000). Strategies that allow plants to survive freezing temperatures have been placed into two major categories: those plants that exhibit deep supercooling characteristics (Ashworth *et al.*, 1993) and those that exhibit extracellular freezing (Burke *et al.*, 1976). Cell dehydration occurs due to this formation of extracellular ice; on the surface of the cell wall, in lumens of non-living fibres and vessels or in the extracellular spaces (Guy, 1990). Liquid water moves out of the cell (Mazur, 1969) and the osmotic concentration inside the cells increases, thus preventing intracellular freezing.

The same phenomenon has been used to explain why the living bark of trees shrinks at freezing temperatures (Wiegand, 1906; Winget and Kozlowski, 1964). Although this mechanism was reported in the 19th century (Hoffmann, 1857; Sachs, 1860; Friedrich, 1897), it has received little attention until recently (Loris *et al.*, 1999; Zweifel and Häsler, 2000). The latter authors showed reversible shrinkage of bark of mature subalpine conifers

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and concluded there was transport of water between bark and wood and that, when ice crystals melted, water returned to the living cells of the bark.

Plants of temperate zones undergo cyclic change in freezing tolerance each year. Classically, cold acclimation with the cessation of growth in the autumn is initiated by certain environmental stimuli (e.g. decreased daylength, changes in light quality, decreasing temperature, and drought stress) and during the spring, plants begin to deacclimate (Bowers, 1994). In contrast, non-acclimated plants have cell death at freezing temperatures, caused by disruption of cell membranes and other cellular components (Burke *et al.*, 1976; Steponkus, 1984). This disruption is usually manifested as a flaccidity and/or discoloration of the affected tissues (Burke *et al.*, 1976; Rodrigo, 2000).

In the present study, apparent extracellular freezing was observed by stem diameter changes on orchard trees of walnut in the field. To evaluate frost acclimation, potted trees or stem segment were submitted to various freezing experiments in a climatic chamber. The authors hypothesize that (1) for acclimated plants, similar reversible shrinkage of bark would be observed in orchard trees, potted trees and isolated stem segments; (2) compared to acclimated trees, non-acclimated trees would show no reversible shrinkage of bark due to cell death and water loss; and (3) with autoclave treatments to cause cell death, no shrinkage event would be observed with freeze events because no differential ice crystal formation would take place. The determination of how much of the stem shrinkage could be attributed to changes within the bark tissue, that is, changes that were independent of the radial supply of water from the xylem, was also desirable.

Linear variable differential transformers (LVDT sensors) have been successfully used as a non-invasive method to detect the water status of plants (Klepper *et al.*, 1971; McBurney and Costigan, 1984; Garnier and Berger, 1985; Wronski *et al.*, 1985; Cochard *et al.*, 2001). In testing the above hypotheses with regard to freezing, it is possible that LVDTs could also offer a novel approach to the detection of freezing and freezing damage in plants.

Materials and methods

In the field and throughout various experiments stem diameter variations were continuously monitored with LVDT devices (models DF 2.5 and DF 5, Solartron Metrology, Massy, France) with sensitivity ± 1 µm. Stem, air, and in some cases soil temperatures were measured with copper–constantan thermocouples and both temperatures and LVDT data were recorded with data loggers (DL2e, Delta T devices, UK). For longer-term field measurements, data were recorded as 1 h averages and averaged at 10 min intervals, whereas for laboratory cooling experiments, data were recorded as 5 min averages and averaged at 1 min intervals.

Temperature and diameter variations measurements were made on excised stems of orchard walnut trees (*Juglans regia* L.

cv. Franquette scions on wild walnut root stocks) in the winters of 1997–98, 1998–99 and 1999–2000. The trees were grown outdoors at the INRA PIAF station near Clermont-Ferrand, in south-central France and they were 17 years old in 1998. Stem segments of twigs were submitted to several freeze–thaw cycles in a temperature-controlled chamber with LVDT sensors, 'Gelista®' (INRA, France). The chamber was designed to hold ten excised stem segments of 5 cm in length and 1 cm in diameter, each with an LVDT device. Cooling and warming cycles were computer-controlled by a circulator bath (Ministat Huber: $-25\ ^\circ$C to $+120\ ^\circ$C) with an external Pt100 into the chamber, with a linear rate of cooling and warming of $5\ ^\circ$C h^{-1}, and with freeze–thaw cycles (0, $-10\ ^\circ$C, 0 $^\circ$C) repeated up to 10 times.

In some cases the bark was neatly peeled, intact, from the stem, down to the vascular cambium. Thus it was possible to compare diameter fluctuations for the following tissues: (1) stem segments with bark on the stem, (2) isolated bark strips (1 cm wide $\times$ 2 cm long), and (3) stem segments with the bark removed. In some cases the stem segment and/or isolated bark was autoclaved to kill all living cells. A minimum of ten replicates for each case were compared with regard to diameter fluctuations during freeze–thaw cycles. As a reference, for one of the aforementioned orchard trees, temperature and diameter variations were measured on the trunk, branches and smaller stems in the field during the winter of 1997–98, in parallel with temperature measurements of the air, trunk, branch, and stem.

In addition to experiments on orchard trees, 5-year-old potted plants of walnut (*Juglans regia* L. cv. Franquette scions on wild walnut root stocks) were used for freezing experiments during the period from October 1998 to the end of May 1999. The grafted plants were grown in individual 33 l well-drained containers filled with a mixture of peat (33%) and clay soil (67%), they were fertilized annually with 10 g NH$_4$NO$_3$ and continuously drip-irrigated to field capacity. Twelve potted trees were grown outdoors until September 1998, when they were put into a 'cool' greenhouse, in which the temperatures were usually kept the same as outdoors. However, air temperatures in the greenhouses were continuously recorded and a heating system was automatically turned on when temperatures dropped to $0\ ^\circ$C, warming the greenhouse to temperatures to as high as $3\ ^\circ$C. Thus the trees were exposed to cool temperatures, but not to sub-zero temperatures, in the autumn and winter of 1998–99, prior to the freezing experiments. As a result, the mean winter temperatures were only slightly higher in the cool greenhouse than outdoors, allowing for possible cold acclimation in the cool greenhouse. A large cooling chamber was designed to hold up to four potted trees that were up to 2 m height. Cooling and warming cycles was computer-controlled and trunk, stem, air, and soil temperature were recorded.

Results

In orchard trees in the winter, the trunk, branches and smaller stems all shrank when temperatures were below $0\ ^\circ$C and expanded as negative temperatures increased back up to $0\ ^\circ$C. Representative results are shown for 10 d in January 1998 in Fig. 1a. This reversible shrinkage was very significant: about 1300 µm, 500 µm and 150 µm for the trunk, branch and stems, respectively when air temperature was at $-10\ ^\circ$C. Little or no diameter fluctuation occurred when the temperatures were above $0\ ^\circ$C during the winter. In the example shown, when the
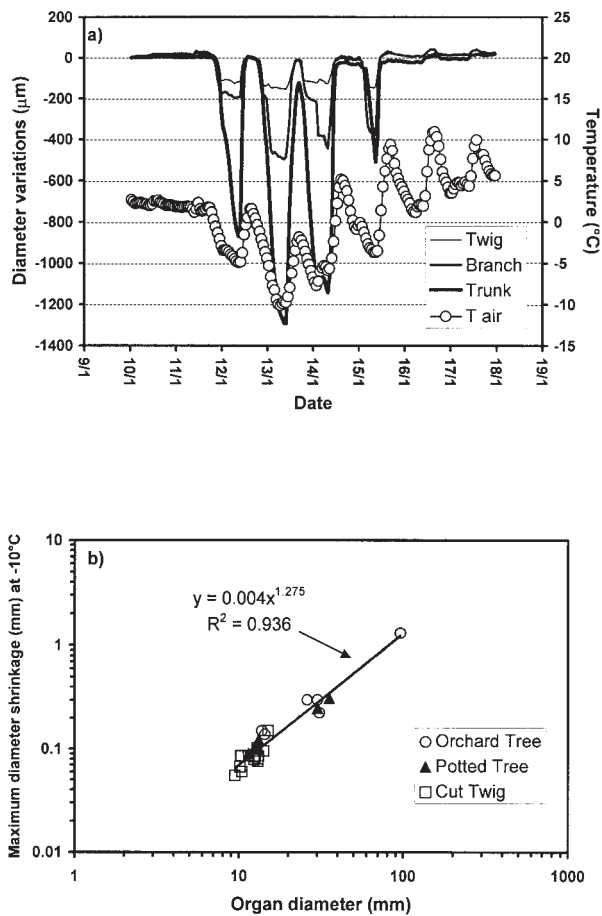
Fig. 1. (a) Diameter variations and air temperature during 10 d in January 1998 for the trunk, a branch and a smaller stem of a walnut tree. (b) Maximum diameter shrinkage at −10 °C versus organ diameter for the same time period and for the orchard tree ($n = 6$), for potted trees ($n = 6$) and isolated stem segments ($n = 13$).

Fig. 2. Stem diameter with progressive freeze–thaw cycles, with decreases in minimum temperature every two cycles. Stem shrinkage was observed only when stems presented an exotherm, at about −6 °C). (a) In early November 1999, the diameter fluctuations were not entirely reversible and, repeated freeze–thaw cycles resulted in progressive loss of diameter. (b) In late November 1999 large and mostly reversible diameter fluctuations were observed.

temperatures stayed near +2 °C for 2 d (10/1 and 11/1) or with positive temperature between 0 to +11 °C for 3 d (16/11 to 18/1), there was little diameter fluctuation. The magnitude of the reversible shrinkage was proportional to the diameter of the stem, regardless of whether the stem was on an intact orchard tree, a potted tree, or isolated stem segments (Fig. 1b).

With the Gelista™ chamber, progressive freezing temperatures (−2.5, −5, −7.5, −10 °C) were imposed on isolated stem segments from orchard trees in early (Fig. 2a) and late (Fig. 2b) November 1999. The cooling cycles with minimum temperatures from −2 to −4 °C resulted in no diameter changes. Shrinkage was observed only when exotherms appeared, which indicated a freezing event had occurred within the stem. In isolated stem segments these would typically occur at temperatures around −5 °C to −8 °C.

The LVDT results were quite different in early (Fig. 2a) versus late (Fig. 2b) November, suggesting an acclimation response. F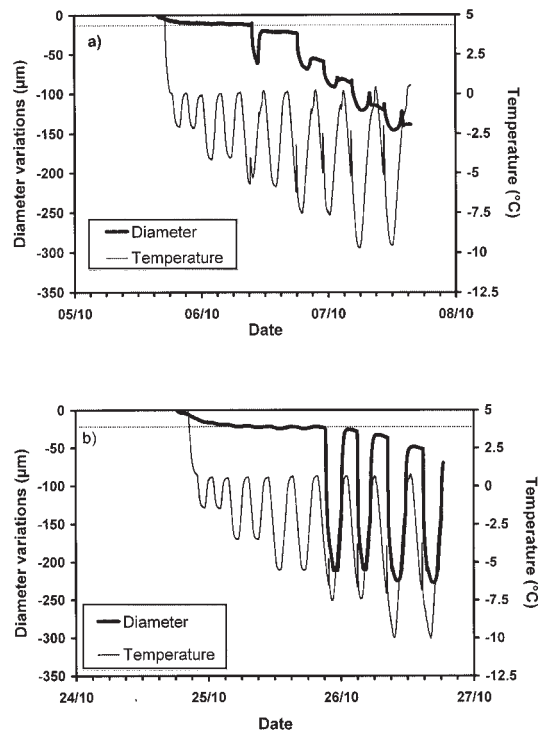or instance, during early November, the diameter fluctuations were not entirely reversible, and repeated freeze–thaw cycles resulted in progressive loss of diameter. Furthermore, after repeated freeze–thaw cycles, there were spikes (transient increases) in stem diameter associated with exotherms and endotherms (Fig. 2a). In contrast, in late November, there were large, reversible declines in diameter that were initiated with the freeze events, and, even with many freeze–thaw cycles, there were no positive spikes in diameter associated with the exotherm or endotherm events (Fig. 2b).

Cooling experiments with minimum temperatures of −10 °C always resulted in exotherms and stem diameter fluctuations. This was true for both the potted trees in the large cooling chamber (Fig. 3c, d) and for isolated stems segments from the orchard trees cooled in the Gelista™ chamber (Fig. 3a, b). With both experimental systems, during the winter (Fig. 3b, d), freeze–thaw cycles resulted in reversible declines in stem diameter. In contrast, during the autumn (Fig. 3a, c), much of the loss in diameter was irreversible, and, after the first freeze–thaw event, there were transient spikes in diameter associated with each exotherm and endotherm.

In Fig. 4 the contribution of different parts of the stem to the diameter variation with a freeze–thaw cycle is quantified. Results are shown for an entire stem segment,
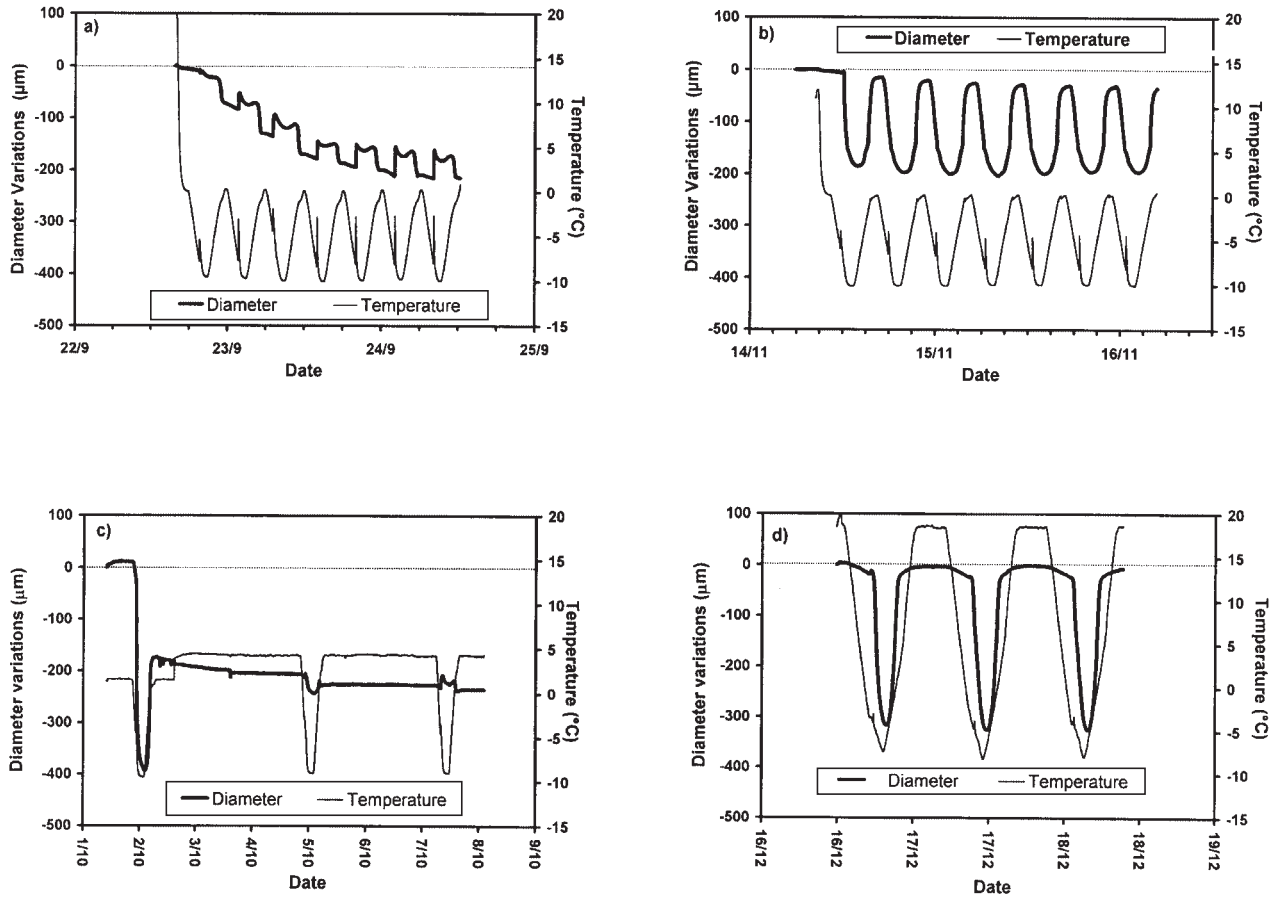
Fig. 3. Stem diameter variations and stem temperature during several freeze–thaw cycles for a stem segment (a) in September 1998, and (b) in November 1998. Trunk diameter variations and trunk temperature and for a potted tree (c) in October 1998 and (d) in December 1998.

a stem with the bark removed to expose the xylem (wood), and for an isolated strip of bark (Fig. 4a). In this example only 14% of the stem diameter change can be explained by fluctuations in the wood diameter. The bark strip represented 43% of the diameter shrinkage. However, considering that an entire stem has bark on each side, simulated stem diameter variation can be calculated as $2 \times$ bark + wood. Results for the simulated stem diameter variation were very similar to those for the intact stem. A similar comparison ($2 \times$ bark versus stem: $n = 16$) was repeated for stems of various diameters in Fig. 4b. By this analysis, diameter variation due to bark shrinkage represented 71% of the total stem maximum shrinkage.

In Fig. 5a, diameter fluctuations for three freeze–thaw cycles were observed for an acclimated stem segment, and a similar autoclaved stem segment. An exotherm with each stem freezing event (arrows) was observed at temperatures below -4 °C. At the exact time of the exotherm, stem shrinkage was observed for the control stem. In contrast, stem swelling was observed for the autoclaved stem. Considering again that an entire stem has bark on each side, the simulated stem diameter variation for

autoclaved stems was calculated as $2 \times$ bark. Simulated autoclaved stem diameter variation ($2 \times$ autoclaved bark) matched the diameter variation for the entire autoclaved stem segment quite well (Fig. 5b)

Discussion and conclusions

In these studies, based upon the exotherms, stem freezing occurred at temperatures between -4 °C to -8 °C. This shows that some supercooling did occur, although it was not a deep supercooling (Ashworth *et al.*, 1993). The diameter variations associated with the freeze–thaw cycles were similar for orchard trees, potted plants in large cooling chambers and isolated stem segments in the Gelista[®] chamber. Quantitatively, the maximum shrinkage appears related with stem diameter, probably due mostly to differences in the thickness of the bark in different size stems.

During the summer season LVDT devices have been used to observe changes in stem diameter that related to variations in water content (Améglio and Cruiziat, 1992; Simonneau *et al.*, 1993; Zweifel *et al.*, 2000) and it can be
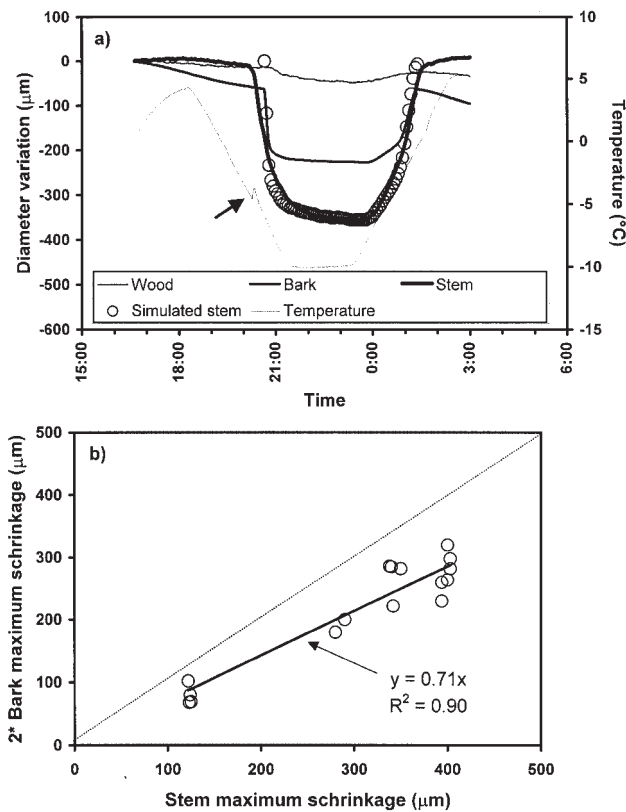
Fig. 4. (a) Diameter variation during one freeze–thaw cycle for an acclimated stem segment, a stem segment with the bark removed, leaving only wood, a bark segment, and a simulated stem segment (2 barks + 1 wood). The arrow indicates an exotherm. (b) Maximum stem segment shrinkage at −10 °C versus 2× maximum bark shrinkage at −10 °C for different sizes of stem ($n = 16$).

Fig. 5. Diameter variations (a) during three freeze–thaw cycles for an acclimated stem segment and for an autoclaved stem segment. (b) Diameter variations during one freeze–thaw cycle for an autoclaved stem segment and for 2× autoclaved bark segment. Autoclaved bark temperature is also represented. Arrows indicate exotherms.

seen that a similar approach could be applied to observing freeze–thaw cycles. Consistent with results presented by Zweifel and Häsler for conifers (Zweifel and Häsler, 2000), the wood of walnut showed only small variations (about 15% of the total stem diameter change) with the freeze–thaw cycle. Such results are similar to those for summer diurnal change in stem diameter, where wood contributes a maximum of 10% of the trunk diurnal shrinkage (Molz and Klepper, 1973; Siau, 1984; Huguet, 1985).

However, the present results are contrary to results in *Picea abies* (Zweifel and Häsler, 2000), where the changes in bark thickness occurred only when the bark was in contact with xylem. For walnut, water transport between the bark and wood was not necessary for most of the freeze-induced shrinkage to occur. Indeed, similar frost shrinkage was observed on portions of isolated bark as with intact stems. In this process, extracellular water freezes first since it has a lower solute concentration than intracellular vacuolar and cytoplasmic water. Once the temperature drops below the freezing point, the vapour deficit will be higher than that of the extracellular ice at the same temperature (Mazur, 1969; Loris *et al.*, 1999).
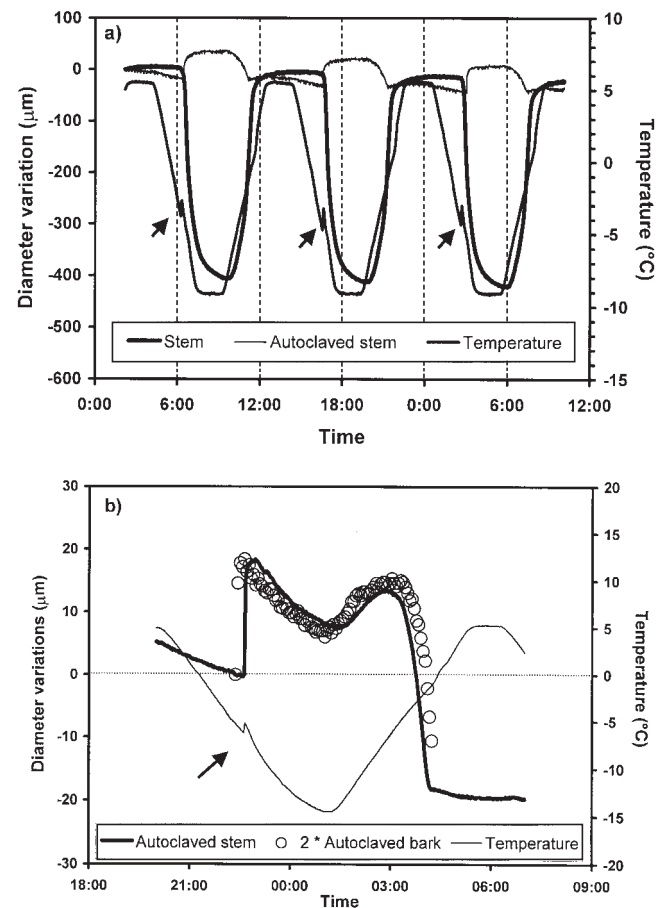
Consequently, water diffuses from the cells through the plasma membrane to the ice crystals in the extracellular matrix and, as a consequence, cell water is lost. However, the water lost by cells due to freezing in extracellular spaces should increase by about 9% in volume by changing state. Why, in these conditions, is a decrease observed rather than an increase in the thickness of the bark? The reason could be that the structure of the bark is very porous. In this case, air zones, retracting with the decrease in temperature, would fill with ice, without adding to the thickness of the bark, while the living cells shrink due to water loss. A Cryo-SEM study will have to be undertaken to understand the location of the ice further and to explain why, in the present results with walnut, a contraction of the bark was observed even when it was isolated from the rest of the stem. For mature subalpine conifers, the same contraction was observed only when the bark was in contact with the wood (Zweifel and Häsler, 2000).

Considering the amount of stem shrinkage associated with summer drought in walnut, the amount of

contraction of the bark with freezing is actually much less than might be predicted by water relations theory. A combination of water relations theory and the Clausius-Claperyron equation was employed to derive a relationship that describes the water potential of extracellular ice and supercooled water in the cell and the water potential of ice at any given freezing temperature (Rajashekar and Burke, 1982; Rajashekar *et al.*, 1983):

$$\Psi_T \text{ (ice)} = 1.16 \text{ (}^{\circ}\text{C)} \tag{1}$$

with Ψ_T (ice) as the water potential (MPa) of ice at temperature T (°C), which is the temperature below freezing. Thus the decline in the water potential of the ice with decreasing temperature is very large, -1.16 MPa °C^{-1} (Guy, 1990). Thus the water potential of the ice at -10 °C would be -11.6 MPa, which represents a drastic water stress, much greater than the water deficits that occur in walnut trees during summer. For example, at -1.5 MPa in walnut, embolisms appeared in leaf petioles and at -2.5 MPa, embolism was total in the stem (Tyree *et al.*, 1993; Cochard *et al.*, 2000). However, during wintertime, the xylem pathway can be also blocked by embolisms that occur as a consequence of the freeze–thaw cycles (Zimmerman, 1983; Cochard and Tyree, 1990; Just and Sauter, 1991; Améglio *et al.*, 1995; Pockman and Sperry, 1997). When the xylem sap freezes, previously dissolved gases form bubbles due to their very low solubility in ice (Sperry and Sullivan, 1992). Upon thaw, these bubbles can either dissolve back into the xylem sap or they can grow to obstruct the entire xylem conduit by embolism (Yang and Tyree, 1992).

Considerable evidence confirms that larger xylem vessels are more vulnerable to embolism by freezing than smaller vessels and tracheids (Hammel, 1967; Succoff, 1969; Ewers, 1985; Sperry and Sullivan, 1992; LoGullo and Salleo, 1993; Hacke and Sauter, 1996; Davis *et al.*, 1999) and the principal explanation is that larger xylem vessels produced large bubbles, for which embolism reversal is difficult (Yang and Tyree, 1992). Nevertheless, it was shown in *Fagus sylvatica* L., that the likelihood of embolism formation is more dependent on the dynamics of sap freezing than xylem characteristics (Lemoine *et al.*, 1999). Perhaps the large gradient of water potential due to ice has often been overlooked and, if the thawing is not homogeneous in the xylem, the large gradients of water potential due to ice may increase the risk of cavitation.

When, for the same diameter stems, shrinkage due to summer water stress versus that due to winter freezing is compared, there are similar diameter fluctuations for very different water potentials. As an example, it has been observed for shoot segments with 10–15 cm diameter, 200 μm diameter shrinkage for a loss in water potential of -1.0 MPa (Cochard *et al.*, 2001). Similar shrinkage can be seen in Fig. 2b for a freeze event to -10 °C, which should correspond to a water potential loss of -11.6 MPa. It may be that resistance to water flow prevents equilibrium in the case of freeze events. Two hypotheses could explain this point: (1) after the initial water lost due to the ice nucleation in the extracellular space, extracellular ice stops water flow and/or (2) due to cellular plasmolysis with freeze events, resistance to water flow increases greatly.

Figure 2 supports these possible interpretations. When progressive freezing temperatures (-2.5, -5, -7.5, -10 °C) were imposed on a stem, shrinkage was observed only when the exotherm appeared, at -6 °C, for 2 cycles at -7.5 °C and 2 cycles at -10 °C. A similar maximum diameter shrinkage (about -225 μm) was observed even though there was a theoretical water potential difference of -2.9 MPa between -7.5 °C and -10 °C, based upon equation (1).

As hypothesized, reversible stem shrinkage was obtained for living tissues, but not for autoclaved tissue. In this case, swelling was observed with the freeze and this swelling could be explained by bark alone, and due to water expansion with the liquid solid phase change. Similar swelling was observed during September for isolated stem segments and in October for intact potted plants (cf. Fig. 2a and c). By comparison with autoclaved treatment, this result was analysed in terms of cell death due to non-acclimated trees. In this case, freezing injury to the bark, with discoloration of tissues, was observed after few cycles at the same temperature.

Given that the diameter fluctuation patterns were dramatically different for acclimated versus non-acclimated plants, and for living versus autoclaved tissues, LVDT sensors could represent a novel, non-invasive approach to the testing of cold hardiness (Améglio *et al.*, 2001). It presents the advantage of being sparing of plant material, allowing its use in breeding programmes, and similar results were obtained for intact plants as for isolated bark, making the technique well adapted to the field or laboratory.

Acknowledgements

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